Exhibit 7

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Original Contributions

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Case Report

Comments, Opinions, and Reviews

Letters to the Editor

Abstracts of Literature

Use of a Spectrophotometric Hemoglobin Assay to Objectively Quantify Intracerebral Hemorrhage in Mice

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Background and Purpose There is great interest in developing novel anticoagulant or thrombolytic strategies to treat ischemic stroke. However, at present there are limited means to accurately assess the hemorrhagic potential of these agents. The present studies were designed to develop and validate a method to accurately quantify the degree of intracerebral hemorrhage (ICH) in murine models.

Methods In a murine model, ICH was induced by stereotaxic intraparenchymal infusion of collagenase B alone (6×10^{-6} U; n=5) or collagenase B followed by intravenous recombinant tissue plasminogen activator (rt-PA) (0.1 mg/kg; n=6). Controls consisted of either sham surgery with stereotaxic infusion of saline (n=5) or untreated animals (n=5). ICH was (1) graded by a scale based on maximal hemorrhage diameter on coronal sections and (2) quantified by a spectrophotometric assay measuring cyanomethemoglobin in chemically reduced extracts of homogenized murine brain. This spectrophotometric assay was validated with the use of known quantities of hemoglobin or autologous blood added to a separate cohort of homogenized brains. With this assay, the degree of hemorrhage after focal middle cerebral artery occlusion/reperfusion was quantified in mice treated with postocclusion high-dose intravenous rt-PA (10 mg/kg; n=11) and control mice subjected to stroke but treated with physiological saline solution (n=9).

Results Known quantities of hemoglobin or autologous blood added to fresh whole brain tissue homogenates showed a linear relationship between the amount added and optical density (OD) at the absorbance peak of cyanomethemoglobin (r=1.00 and .98, respectively). When in vivo studies were performed to quantify experimentally induced ICH, animals receiving intracerebral infusion of collagenase B had significantly higher ODs than saline-infused controls (2.1-fold increase; P=.05). In a middle cerebral artery occlusion and reperfusion model of stroke, administration of rt-PA after reperfusion increased the OD by 1.8-fold compared with animals that received physiological saline solution (P<.001). When the two methods of measuring ICH (visual score and OD) were compared, there was a linear correlation (r=.88). Additional experiments demonstrated that triphenyltetrazolium staining, which is commonly used to stain viable brain tissue, does not interfere with the spectrophotometric quantification of ICH.

Conclusions These data demonstrate that the spectrophotometric assay accurately and reliably quantifies murine ICH. This new method should aid objective assessment of the hemorrhagic risks of novel anticoagulant or thrombolytic strategies to treat stroke and can facilitate quantification of other forms of ICH. (***Stroke.*** **1997;28:2296-2302.)**

Key Words • anticoagulants • intracerebral hemorrhage • plasminogen activator, tissue-type • mice

Ischemic stroke accounts for the greatest majority of presentations of acute stroke. There has therefore been a tremendous interest in designing strategies that can promptly and effectively restore blood flow to the ischemic region of brain. Although heparin may be effective in incipient stroke (TIAs),[1] its use during the acute phases of stroke may be associated with a high degree of morbidity and ICH.[1-4] Similarly, in the early 1960s, the dismal outcomes in the streptokinase trials for acute stroke led to the reluctance of clinicians to use thrombolytic therapy for acute stroke for the subsequent three decades.[5,6] This reluctance has been validated by recent trials in which the use of streptokinase has been associated with increased risk of mortality and ICH.[7] On the other hand, the use of rt-PA to treat stroke-in-progress has shown more promise,[8] with a subset of patients with acute stroke who are treated with rt-PA demonstrating reduced long-term morbidity if treated within the first 3 hours of symptom onset.[9-11] Even so, other trials using the same agent (rt-PA) have failed to show benefit or have had excessively high rates of ICH.[9,12-15]

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This confusing morass of clinical data underscores the urgent need to identify improved strategies to achieve rapid reperfusion. Toward this end, it is imperative to identify an experimental model in which the potential benefits of timely reperfusion in stroke can be weighed objectively against the risks of increased ICH. In most animal studies of thrombolytic therapy for clinical stroke, the risks of ICH have been estimated rather than quantitatively measured.[16-24] The present studies were designed to develop and validate a method to accurately quantify the degree of ICH in murine models to assess potential risks of new anticoagulant or thrombolytic treatments for acute stroke.

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Selected Abbreviations and Acronyms

ICH = intracerebral hemorrhage
MCA = middle cerebral artery
OD = optical density
rt-PA = recombinant tissue plasminogen activator
TIA = transient ischemic attack
TTC = triphenyltetrazolium chloride

Materials and Methods

Experimental Animals

In the present study, male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Me) and were used between 8 to 10 weeks of age (weight, 22 to 32 g). All procedures were performed according to an institutionally approved protocol and are in accordance with the guidelines provided by the American Academy of Accreditation of Laboratory Animal Care.

Spectrophotometric Assay for ICH

The hemoglobin content of brains subjected to the experimental procedures below was quantified with a spectrophotometric assay as follows. Whole brain tissue was obtained from freshly killed control or experimental animals, and each brain was treated individually as follows. Distilled water (250 μL) was added to each brain, followed by homogenization for 30 seconds (Brinkman Instruments, Inc), sonication on ice with a pulse ultrasonicator for 1 minute (SmithKline Corp), and centrifugation at 13 000 rpm for 30 minutes (Baxter Scientific Products). After the hemoglobin-containing supernatant was collected, 80 μL of Drabkin's reagent (purchased from Sigma Diagnostics; $K_3Fe(CN)_6$ 200 mg/L, KCN 50 mg/L, $NaHCO_3$ 1 g/L, pH 8.6[25]) was added to a 20-μL aliquot and allowed to stand for 15 minutes. This reaction converts hemoglobin to cyanomethemoglobin, which has an absorbance peak at 540 nm, and whose concentration can then be assessed by the OD of the solution at ≈550 nm wavelength.[25] To validate that the measured absorbance following these procedures reflects the amount of hemoglobin, known quantities of bovine erythrocyte hemoglobin (Sigma) were analyzed with similar procedures alongside every brain tissue assay. As an additional measure, blood was obtained from control mice by cardiac puncture after anesthesia. Incremental aliquots of this blood were then added to freshly homogenized brain tissue obtained from untreated mice to generate a standard absorbance curve.

Collagenase-Induced ICH

The general procedures for inducing ICH in the mouse were adapted from a method that has been previously described in rats.[26] After anesthesia with an intraperitoneal injection of 0.35 mL of ketamine (10 mg/mL) and xylazine (0.5 mg/mL), mice were positioned prone in a stereotaxic head frame. The calvarium was exposed by a midline scalp incision from the nasion to the superior nuchal line, and then the skin was retracted laterally. With a variable-speed drill (Dremel), a 1.0-mm burr hole was made 2.0 mm posterior to the bregma and 2.0 mm to the right of midline. A single 22-gauge angiocathether needle was inserted with stereotaxic guidance into the right deep cortex/basal ganglia (coordinates: 2.0 mm posterior, 2.0 mm lateral). The needle was attached by plastic tubing to a microinfusion syringe, and solutions were infused into the brain at a rate of 0.25 μL/min for 4 minutes with an infusion pump (Bioanalytical Systems). Animals received either (1) 0.024 μg collagenase B (Boehringer Mannheim) in 1 μL normal saline solution (collagenase group); (2) 1 μL normal saline solution alone (sham group); (3) no treatment (control group); or (4) stereotaxically-guided infusion of collagenase B as above but followed immediately by intravenous rt-PA (Genentech Inc; 1 mg/kg in 0.2 mL normal saline solution) administered by dorsal penile vein injection (collagenase+rt-PA group). In the collagenase, sham, and collagenase+rt-PA groups, the stereotaxic needle was removed immediately after infusion, and the incision was closed with surgical staples. Brain tissue was harvested immediately after rapid anesthetized decapitation.

Hemorrhagic Conversi n in a Murine Focal Cerebral Ischemia Model

Focal cerebral ischemia was produced in animals by transient right MCA occlusion according to a method previously described in detail.[27,28] Briefly, a heat-blunted 12- or 13-mm 5–0 or 6–0 gauge nylon suture was passed into the right internal carotid artery to the level of the MCA. After 45 minutes, the occluding suture was removed to reestablish perfusion. Immediately after removal of the occluding suture, animals received either intravenous rt-PA (10 mg/kg in 0.2 mL normal saline solution; stroke+rt-PA group) or normal saline solution (stroke+saline group) given by dorsal penile vein injection. At 24 hours, brain tissue was harvested immediately after rapid anesthetized decapitation. To evaluate the effect of TTC, which is commonly used to distinguish infarcted from noninfarcted cerebral tissue,[27,29] nonmanipulated (control) brains were divided in half, immersed in 2% TTC (Sigma Chemical Co) in 0.9% phosphate-buffered saline, incubated for 30 minutes at 37°C, and then prepared as described above for the spectrophotometric hemoglobin assay. The other half of each brain was immersed in saline for an identical duration and then subjected to the procedures described above for the spectrophotometric hemoglobin assay.

Validation of Quantitative ICH Assay

The degree of ICH was first scored visually by a blinded observer. For visual scoring of ICH in mice, brains obtained from mice that had survived to the 24-hour time point after the procedure (collagenase-induced hemorrhage or MCA occlusion) were placed in a mouse brain matrix (Activational Systems Inc) to obtain 1-mm serial coronal sections. Sections were inspected by a blinded observer, and brains were given an ICH score from a graded scale based on maximal hemorrhage diameter seen on any of the sections (ICH score 0, no hemorrhage; 1, <1 mm; 2, 1 to 2 mm; 3, >2 to 3 mm; and 4, >3 mm). Slices from each brain were then pooled, homogenized, and treated according to the procedures described above for the spectrophotometric hemoglobin assay.

Statistical Analysis

Correlations between visually determined ICH scores and spectrophotometric determinations of ICH were performed with the use of Pearson's linear correlation, with correlation coefficients indicated. To establish whether a given treatment (eg, collagenase, sham, stroke) had a significant effect on either spectrophotometric or visually scored ICH, comparisons were made with an unpaired two-tailed t test. For nonparametric data (visual ICH scores), nonparametric analysis was performed with the Mann-Whitney test. Values are expressed as mean±SEM, with $P<.05$ considered statistically significant.

Results

Spectrophotometric Hemoglobin Assay

Initial studies were performed to determine the reliability and reproducibility of the spectrophotometric hemoglobin assay. In the first set of experiments, known quantities of hemoglobin were converted to cyanomethemoglobin according to previously published procedures, and the OD was measured (Fig 1A).[25] In a second set of experiments, known quantities of autologous blood were added to fixed volumes of fresh brain tissue homogenate, with further treatment of specimens as described above. These data show that the OD of

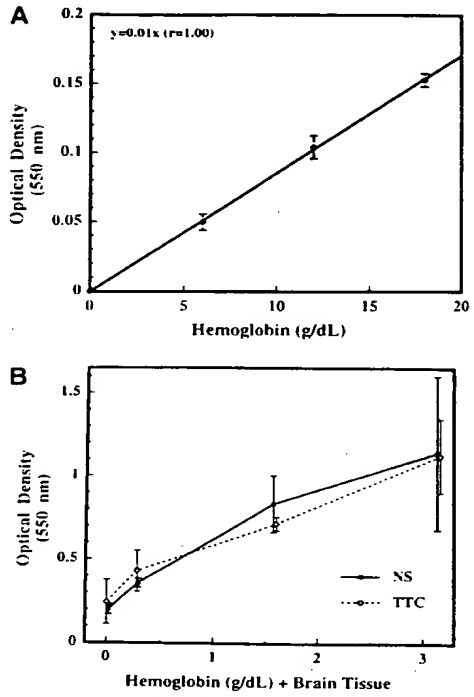

FIG 1. Validation of quantitative spectrophotometric ICH assay in the absence (A) or presence (B) of brain tissue. A, Standard curve in which known concentrations of hemoglobin were reduced to cyanomethemoglobin, after which the OD at 550 nm was measured. Mean±SEM values are shown; n=5 determinations at each point. The equation for the best-fit line and r value are shown. B, Known concentrations of hemoglobin (with the use of autologous blood diluted in saline) were added to fixed volumes of fresh brain tissue homogenate, and the spectrophotometric hemoglobin assay was performed. Brains were divided into hemispheres; for each animal, one hemisphere was immersed in physiological saline for 20 minutes (NS), and the other hemisphere was placed in TTC for 20 minutes (similar to the procedure that would be done to measure cerebral infarction volume). For each concentration of added hemoglobin, spectrophotometric hemoglobin assay was performed on six hemispheres. Mean±SEM values are shown.

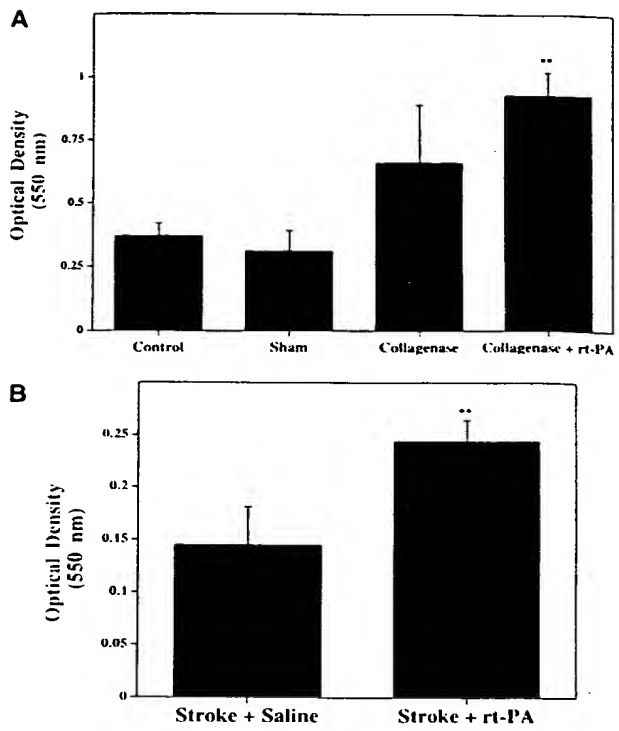

FIG 2. Quantitative spectrophotometric hemoglobin assay. A, Effects of collagenase-infusion and rt-PA on murine quantitative ICH. Mice were stereotaxically infused with ICH-inducing agents into the right deep cortex/basal ganglia. Brains were harvested 24 hours later, and the spectrophotometric hemoglobin assay was performed to quantify ICH. Mice were subjected to (1) no treatment (Control), (2) stereotaxic infusion of 1 μL normal saline solution (Sham), (3) stereotaxic infusion of 0.024 μg collagenase B in 1 μL normal saline solution (Collagenase), or (4) stereotaxic infusion of collagenase B (as above) followed by intravenous tissue plasminogen activator (1 mg/kg in 0.2 μL normal saline solution) by dorsal penile vein injection (Collagenase+rt-PA). $^{**}P<.001$ vs sham or control. B, Effect of rt-PA after focal ischemic stroke on murine quantitative ICH. Mice were subjected to 45 minutes of MCA occlusion followed by reperfusion and then (1) intravenous 0.2 μL of normal saline solution (Stroke+Saline) or (2) intravenous rt-PA (15 mg/kg in 0.2 μL normal saline solution) (Stroke+rt-PA). Brains were harvested 24 hours later, and the spectrophotometric hemoglobin assay was performed to quantify ICH. $^{**}P<.05$.

cyanomethemoglobin-containing supernatants at 550 nm correlated linearly with the amount of added blood (Fig 1B). These data show tight linear correlations ($r=1.00$ and .98 for Fig 1A and 1B, respectively), as well as excellent reproducibility as gauged by relatively small standard errors of the mean. To establish that TTC (commonly used to distinguish infarcted from noninfarcted cerebral tissue[27,29]) does not affect the spectrophotometric hemoglobin assay, nonmanipulated (control) brains were divided in half, with half being subjected to the standard TTC staining procedure and half being treated with saline as a control. These data (compare Fig 1B, solid and dashed lines) indicate that pretreatment of brain tissue with TTC does not affect the spectrophotometric hemoglobin assay.

To determine whether this method is able to detect ICH, the assay was performed on murine ICH caused by two different procedures, intraparenchymal collagenase infusion or MCA occlusion/reperfusion. In the first procedure, collagenase B was applied as a local infusion through a burr hole to weaken the vascular wall to promote ICH (collagenase group). To further increase the propensity for and degree of ICH, a similar procedure was performed, with immediate administration of rt-PA after the procedure (collagenase+rt-PA group). Two control conditions were also included: a sham operation that included drilling the burr hole but with instillation of physiological saline (sham), and an untreated group (control). These experiments demonstrated that collagenase infusion increases the amount of intracerebral blood detected by the spectrophotometric assay (especially with collagenase+rt-PA) compared with sham-treated animals or normal controls (Fig 2A).

In the second and perhaps more clinically relevant method for inducing ICH, a stroke was created by transient intraluminal occlusion of the MCA followed by reperfusion. In addition, we attempted to increase the propensity for hemorrhagic conversion by administration of a thrombolytic agent. Two groups were studied:

those that had received normal saline solution and those that received intravenous rt-PA immediately after removal of the intraluminal occluding suture. These data indicate that the addition of a fibrinolytic agent after stroke increases the amount of ICH that is detected by the spectrophotometric hemoglobin assay (Fig 2B). It is interesting to note that baseline absorbance is lower in animals subjected to stroke than control/untreated animals (Fig 2A and 2B). To further investigate how residual intravascular blood might affect the spectrophotometric hemoglobin assay, experiments were performed in which, immediately before decapitation of the animal for brain harvest, a cephalic perfusion of physiological saline was performed (administered through the left cardiac ventricle). In control animals (n=5), which received cardiac saline perfusion before brain harvest, the mean OD after tissue preparation and spectrophotometric hemoglobin assay was 0.25±0.3 (lower than the OD seen in noncardiac perfused animals subjected to either no or sham surgery) (n=10; OD, 0.34±0.05; P=.05 versus cardiac perfused controls). In contrast, after stroke there was no difference in OD whether or not cardiac saline perfusion was performed (0.15±0.04 for stroke without cardiac saline perfusion, n=5; 0.15±0.03 for stroke with cardiac saline perfusion, P=NS). When saline-perfused animals with stroke were compared with saline-perfused animals without stroke, there was an apparent reduction in OD after spectrophotometric hemoglobin assay. These data suggest that animals with a stroke have less intracerebral blood detected, perhaps as the result of a reduction of the total amount of blood in the ipsilateral MCA region after ischemia.

Visual ICH Score

To further validate the spectrophotometric hemoglobin assay, we compared it with morphometric assessment of hemorrhage size, which has traditionally been used in the literature.[30-34] We developed a visual scoring system (0 to 4) in which a blinded observer scored the degree of ICH in serial cerebral sections based on maximal hemorrhage diameter. This visual assessment was performed on a photograph of the brain taken immediately before the performance of the spectrophotometric hemoglobin assay (Fig 3), so that the two techniques could be correlated on the same specimens. When compared with controls not subjected to any intervention, animals receiving a sham local infusion (ie, burr hole+saline) demonstrated only a slight increase in visual ICH score (Fig 4A). However, when either collagenase alone or collagenase+rt-PA was added to the infusate, visual ICH scores were significantly increased (Fig 4A). In the stroke model, rt-PA similarly resulted in an increase in the visual ICH score (Fig 4B). When the data are plotted to show the relationship between the visual ICH score and the spectrophotometric technique for quantifying ICH, a linear relationship was suggested (r=.88); however, with smaller degrees of hemorrhage (visual ICH scores of 0 or 1), this relationship did not hold (Fig 5).

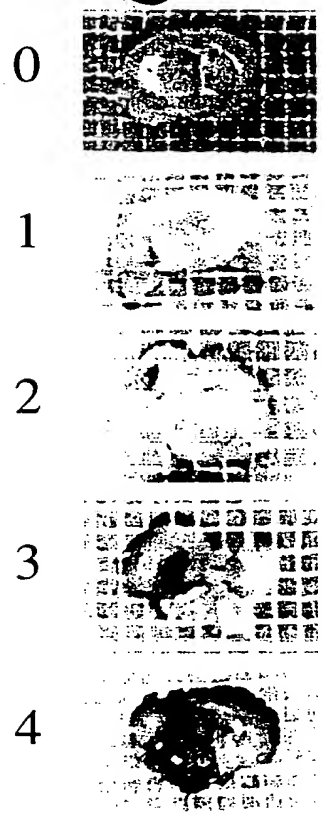

FIG 3. Demonstration of the scoring system used for the visual determination of ICH after stroke. Each slice, taken from different animals subjected to stroke, represents the coronal slice of brain that exhibits the maximal hemorrhagic diameter. The numbers correspond to the visually determined hemorrhage score, as defined in "Materials and Methods."

Discussion

Recently, it has become apparent that early intervention in stroke with certain intravenous thrombolytic agents (rt-PA) may be beneficial if instituted within 3 hours of symptom onset.[9,10] However, administration of thrombolytic agents outside of this narrow therapeutic window can cause an unacceptably high incidence of devastating ICH (streptokinase versus placebo: 10-day mortality, 34.0% versus 18.2%, P=.002; 6-month mortality, 73% versus 59%, P=.06).[7] It therefore remains a clinical imperative to identify more optimal agents for restoring perfusion that are associated with less risk of hemorrhagic conversion. To adequately study new agents that interfere with coagulation or fibrinolytic mechanisms, it is necessary to have an objective means of quantifying the risk of ICH. In the experimental literature, quantification of ICH has been performed either by radiological imaging procedures[31-36] or by a visual estimation of the amount of hemorrhage in postmortem brain tissue.[30-34] These procedures are of limited usefulness depending on the conditions under study. For instance, in addition to the logistic constraints imposed by the need for sophisticated equipment, most radiological imaging techniques are of limited use in murine models, which may preclude their use in the evaluation of transgenic mice, a potentially powerful tool for studying the coagulation or fibrinolytic systems. Visual estimation of ICH is subjective in nature and, as our own data show, may be relatively insensitive for detecting small degrees of ICH. Furthermore, neither the radiological nor the visual techniques permit accurate quantification of ICH when the hemorrhagic region is patchy or multifocal.

The present studies were performed to develop and validate an objective method for quantifying ICH in experimental animals. The use of a spectrophotometric assay for the quantification of hemoglobin based on the

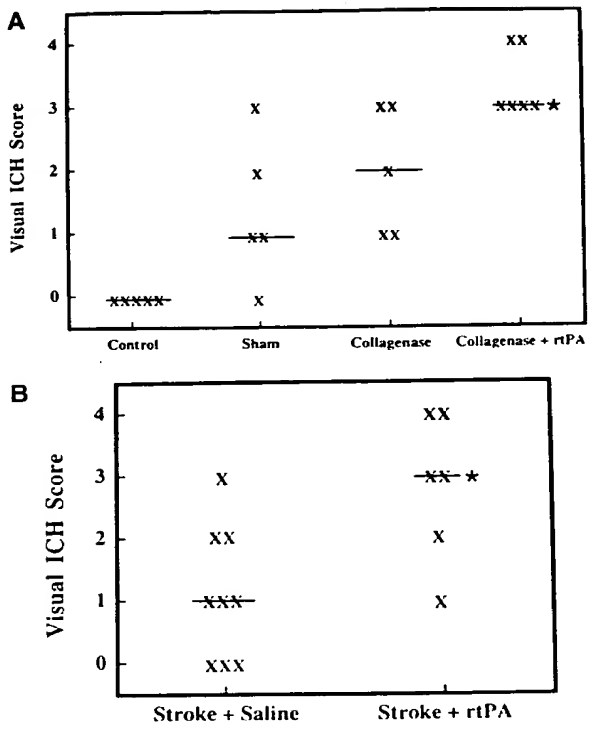

FIG 4. Visual ICH score. A, Effects of collagenase infusion and rt-PA on murine visual ICH score. Mice were stereotaxically infused with ICH-inducing agents into the right deep cortex/basal ganglia. Mice were subjected to (1) no treatment (Control), (2) stereotaxic infusion of 1 μL normal saline solution (Sham), (3) stereotaxic infusion of 0.024 μg collagenase B in 1 μL normal saline solution (Collagenase), or (4) stereotaxic infusion of collagenase B (as above) followed by intravenous tissue plasminogen activator (1 mg/kg in 0.2 μL normal saline solution) by dorsal penile vein injection (Collagenase+rt-PA). Brains were harvested 24 hours later, sectioned into 1-mm coronal slices, and scored by a blinded observer as described in "Materials and Methods." *$P<.05$ vs Collagenase, $P<.005$ vs Sham or Control. B, Effect of rt-PA after focal ischemic stroke on murine visual ICH score. Mice were subjected to 45 minutes of MCA occlusion followed by reperfusion and then (1) intravenous 0.2 μL of normal saline solution (Stroke+Saline) or (2) intravenous rt-PA (15 mg/kg in 0.2 μL normal saline solution) (Stroke+rt-PA). Brains were harvested 24 hours later, sectioned into 1-mm coronal slices, and scored by a blinded observer as described in "Materials and Methods." *$P<.01$. Individual values for visual ICH scores are shown, with the median value for each group indicated by a horizontal line.

conversion of hemoglobin to cyanomethemoglobin has been previously reported.[25,30] However, to the best of our knowledge, in the brain it has only been used in rats to measure the size of a frank blood clot after its removal from adjacent brain tissue.[30] The spectrophotometric assay we describe and validate can be used in animals as small as mice, which facilitates the use of the many transgenic mouse strains now available (particularly those with alterations in the thrombotic or fibrinolytic cascades). Furthermore, this spectrophotometric assay permits the quantification of ICH even when there are patchy or multifocal hemorrhages, which would be otherwise difficult to identify or isolate. Finally, in contrast to the study of Lee et al,[30] we have validated our study for reproducibility and reliability using known quantities of hemoglobin and autologous blood admixed with brain

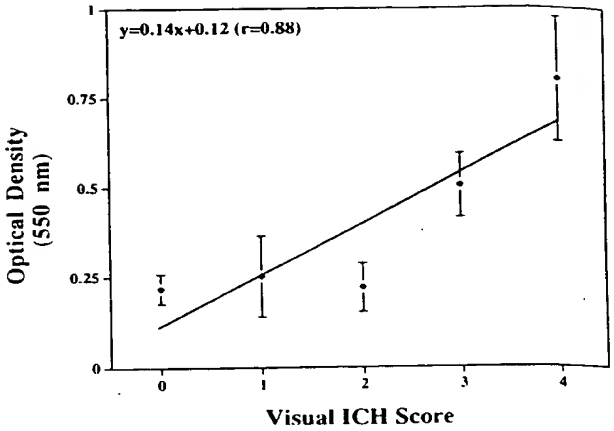

FIG 5. Correlation between visual ICH and spectrophotometric hemoglobin assay. OD at 550 nm (ordinate) represents the results obtained from the spectrophotometric hemoglobin assay in which brain tissue (from all experiments) was analyzed. The corresponding visual ICH scores (as shown in Fig 4) are plotted along the abscissa. For each point, mean±SEM values are shown. Linear correlation was performed with Pearson's linear correlation, with the equation of the line and r value shown.

tissue. Because the surgical procedure used in the stroke experiments did not significantly alter blood hemoglobin concentrations (data not shown), the spectrophotometric hemoglobin assay may be used to extrapolate the volume of ICH when the hemoglobin concentration is known at the time of hemorrhage.

To develop and validate the spectrophotometric hemoglobin assay for situations that may be relevant for clinical ICH, we created ICHs by two different methods: (1) intracerebral injection of collagenase (to weaken the vascular wall, as might occur with an aneurysm or with trauma) and (2) a model of stroke. In both instances, a cohort of animals also received rt-PA to validate the model at the high end of the spectrum of ICH. Because there has been no established gold-standard measurement for ICH in mice, our spectrophotometric measurements were compared with ICH size as independently assessed by visual scoring. Finally, to prove the assay even more useful for experimental models of stroke in which brains are stained with TTC to quantify cerebral infarct volume, the brains of animals subjected to MCA occlusion/reperfusion were stained with TTC before pooling and homogenization to establish that the TTC staining procedure itself does not interfere with the ability to quantify ICH by the spectrophotometric hemoglobin assay. These data (Fig 1B) indicate that there is no detectable cross-interference between the two procedures when used sequentially (TTC staining first, followed by homogenization and the spectrophotometric hemoglobin assay).

In addition to its ability to detect ICH, the present studies indicate that this technique may also give an indication of the amount of residual intravascular blood following brain harvest. The procedure of cephalic saline perfusion does not alter the OD for cyanomethemoglobin in brains subjected to stroke, suggesting that the amount of intravascular blood is relatively fixed and does not wash out by the procedure. However, in control animals that have been otherwise untreated, the saline

perfusion treatment does appear to lower the OD for cyanomethemoglobin by approximately 30%. Our experiments do not provide the reason for this difference, but one may speculate that after stroke, there is an element of vasoconstriction/vaso-occlusion in the territory of infarction, which makes the saline perfusion technique less effective at washing out additional residual intravascular blood. Also, if there is truly an element of vasoconstriction after stroke or experimentally induced ICH, this may reduce the intravascular blood pool and hence account for an overall lowering of the OD when control and stroke/ICH brains are compared (even if some extravascular blood is present in the latter group).

Several technical aspects of the spectrophotometric technique for measuring ICH also deserve mention. For the present experiments, although there is a broad absorbance peak for cyanomethemoglobin centered at approximately 540 nm, we measured the absorbance of cyanomethemoglobin at 550 nm. We did this because many spectrophotometers have fixed wavelength capabilities depending on the preset filters, and 550 nm is a commonly used wavelength (especially in enzyme-linked immunosorbent assay plate readers). Although perhaps measurement of absorbance at 540 nm would have yielded slightly higher OD measurements, the absorbance peak of cyanomethemoglobin is broad in this area, and hence 550 nm may be used without the need to correct for the absorbance of ferricyanide or ferrocyanide (the extinction coefficients for cyanomethemoglobin at 551 and 540 nm are 11.5 and 11.1, respectively, compared with the 41-fold lower extinction coefficient of ferricyanide or ferrocyanide[37]). Pilot studies using a continuous wavelength spectrophotometer (which was used to measure OD at 540 nm) and a discrete spectrum enzyme-linked immunosorbent assay plate reader (used to measure OD at 550 nm) gave similar results. Because the latter technique was simpler, increased the throughput of the procedure, and permitted us to minimize sample volume, we elected to use the latter technique for the studies shown in "Results."

Some other potential technical considerations should be considered when the spectrophotometric assay is used. Even though we have shown that the spectrophotometric procedure can be used in conjunction with TTC staining of serial cerebral sections for infarct volume analysis, the tissue must be subsequently homogenized and extracted, destroying tissue architecture and making further histological characterization impossible. It is possible that this technique may overestimate the degree of ICH if extracerebral blood is unintentionally included during brain harvesting, or the technique may underestimate the degree of ICH if residual epidural, subdural, or subarachnoid blood remains adherent to the calvarium, which is discarded during the process of brain removal.

Because of the nature of the measurement technique, in which light at a given wavelength is absorbed along a fixed length path, anything causing turbidity of the homogenized brain supernatant may increase the OD reading. This may include lipids, abnormal plasma proteins, and erythrocyte stroma. In fact, in preliminary experiments we found that ODs were falsely elevated when the centrifugation was insufficient and some of the lipid layer was included in the assay. Free pyridines may alter the absorbance spectrum of cyanomethemoglobin, and there is the potential for other hemochromogens to also react with the Drabkin's reagent.[38] However, to our knowledge these reactions should not interfere to a significant extent with the determination of intracerebral blood/hemorrhage.

In summary, the present data illustrate how a simple and inexpensive spectrophotometric assay for hemoglobin can provide a useful method for quantifying ICH. This technique should prove especially useful to evaluate the hemorrhagic potential of newly developed thrombolytic or anticoagulant therapies for the treatment of stroke.

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Editorial Comment

This is an important methodological study that will certainly be valuable in the assessment of acute hemorrhagic complications from therapeutic intervention in animal models of stroke. Specifically, the authors used murine models of intracerebral hemorrhage and transient focal ischemia as a tool to determine the utility of a spectrophotometric hemoglobin assay to quantify the amount of intracerebral hemorrhage. The data support the hypothesis that the spectrophotometric hemoglobin assay is an accurate tool for determination of the amount of intracerebral hemorrhage after collagenase administration and transient focal ischemia. It is also important that the authors were able to demonstrate that triphenyltetrazolium chloride (TTC) staining had no effect on their measurements. This assay is likely to be frequently used to assess the frequency and intensity of acute hemorrhagic complications in animal models of postischemic reperfusion and thrombolytic therapy.

However, some caution must also be used in interpretation of the data. For example, the comparison method ("gold standard") of scoring the degree of hemorrhage, by the authors' own admission, is very weak. Therefore, even though there is a correlation between the two techniques, the poor gold standard does not independently lend confidence to the hypothesis that the new technique accurately defines the amount of cerebral hemorrhage. With further technological development in subsequent studies the authors may be able to use imaging techniques (eg, MRI) to obtain better spatial and time resolution of hemorrhage volume. Unfortunately, unlike imaging techniques, the technique of whole-brain hemoglobin measurement described in this study allows only for a single snapshot view of brain hemoglobin concentration. Furthermore, the method of tissue processing does no allow for separation of blood from the intravascular and extravascular compartments. Therefore, depending on the effect of a therapeutic intervention on intravascular blood volume, the measurement of total hemoglobin may either overestimate or underestimate the actual hemorrhage volume. Likewise, to estimate hemorrhage volume with the technique of whole-brain hemoglobin measurement described in this study, one would need to know the blood hemoglobin concentration at the actual time of hemorrhage.

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